American Journal of Pathology, Vol. 153, No. 4, October 1998

Commentary

Chronic Inflammation

Links with Angiogenesis and Wound Healing

Guido Majno

From the Department of Pathology, University of Massachusetts Medical School, Worcester, Massachusetts

Late one night in the early days of electron microscopy I found myself staring at an electron micrograph (not mine) in the basement of Rockefeller University. "It is so beautiful," I mumbled, "that it has to be true." "What did you say?" asked a voice with a strong Romanian accent. George Palade did not let me get away with it.

Since then I have been very careful in evaluating the scientific importance of beautiful photographs, although I must confess that occasionally I am still tempted to repeat that controversial statement. A collection of microphotographs that qualify as truly beautiful illustrates a paper in this issue of *The American Journal of Pathology*.[1] It is my privilege to comment on this excellent study by Thurston and colleagues at the University of California at San Francisco.

Oddly enough, there are few microscopic studies of vessels in chronic inflammation and even fewer of vessels in infection. Yet this should be a productive field because some bacteria are directly angiogenic; the vascular component of the wart-like growth called verruga peruana, caused by *Bartonella bacilliformis*, is truly angiomatous.[2,3] In drafting this commentary I decided to let the paper by Thurston et al speak largely for itself. The fact that it links chronic inflammation with angiogenesis gives me the opportunity to broaden the discussion to include fibroblasts and wound healing because, as will be apparent below, these processes are more closely linked than is generally understood. The link is provided by circulating stem cells.

Blood Vessels in Chronic Inflammation

The contribution of blood vessels to inflammation can be divided into two phases. In the first phase, which lasts roughly 24 hours, functional changes prevail: dilatation, increase in permeability, activation of the endothelium, and diapedesis. In the second phase, although some of the functional changes persist, structural changes occur. There is remodeling of vessels, mainly capillaries and venules, with extensive mitotic activity of the endothelium and pericytes. These two phases are generally recognized as "acute" and "chronic," whereby it becomes apparent that biological and clinical notions of "acute" and "chronic" are somewhat out of phase, the biological events being telescoped into a shorter time.[4]

The response of the microcirculation to a chronic stimulus is the issue addressed by the San Francisco group. As a model they chose *Mycoplasma pulmonis* infection of the airways in mice, a model relevant to human disease because *M. pulmonis* belongs to the same genus as the human pathogen *M. pneumoniae*. The authors studied the trachea between 1 and 8 weeks after infection; at each time interval the blood vessels were perfused with silver nitrate or treated by methods that characterize the phenotype of the endothelium (perfusion with suitable lectins, immersion in anti-vW antibody), then the trachea was cut open, flattened out, prepared as a whole mount, and transilluminated. The key feature of this preparation is that it shows the vascular network in three dimensions rather than in cross sections, thus allowing the observer to distinguish capillaries from arterioles and venules. This is important because it is well established that the pathophysiology of the three microvascular segments is very different.

Thurston et al find that the microcirculation responds to the chronic stimulus either by producing more capillaries or by dilating existing ones. Surprisingly, the type of response depends on genes: by testing two strains of mice, characterized by high *versus* low resistance to *Mycoplasma* infection, the authors found that the number of vessels doubled in mice of the resistant strain, whereas existing vessels in susceptible mice became wider. It is not clear whether the difference in vascular response is related to the difference in resistance to infection.

The observed capillary dilatation is more surprising than it may seem because it has been known since Cohnheim (1867)[5] that capillaries in acute inflammation

Accepted for publication June 10, 1998.

Address reprint requests to Guido Majno, M.D., Department of Pathology, University of Massachusetts Medical School, 55 Lake Avenue North, S2–117, Worcester, MA 01655

do not dilate significantly despite the increased blood pressure. Something else must therefore be happening to the capillary wall in chronic inflammation. Perhaps the tone of the endothelial cells is decreased; by growing endothelium on a pliable substrate we have shown that endothelial cells do in fact maintain a normal state of contraction or "tone."[6,7]

With time, the endothelial cells in the dilated capillaries of the inflamed trachea multiply, as we had observed in the rat cremaster.[8] At this point the dilated capillaries acquire functional properties of venules and a sort of metaplasia takes place. We hope to learn from further studies by the San Francisco team whether this change is reversible.

For both new vessels and old ones made larger, Thurston et al report that permeability to Evans blue appears to be in the normal range; however, both types of vessels are more susceptible to a permeability-increasing mediator, substance P. This finding is of interest for the pathophysiology of chronic inflammation as it suggests that newly formed or remodeled vessels could be maintained in an activated and/or leaky condition with doses of chemical mediators too small to affect normal vessels.

Stem Cells, Angiogenesis, and Wound Healing

We naturally think of new blood vessels as deriving from pre-existing blood vessels. However, Thurston et al cite a recent paper[9] that raises another possibility: some endothelial cells may derive from circulating stem cells. This issue needs to be explored. What is known about the possible contribution of stem cells to tissue repair as a source not only of endothelium to support angiogenesis but also of fibroblasts to support wound healing?

The origin of endothelial cells from the blood has been suspected since at least the 1960s.[10,11] One of the first attempts to prove it was that of O'Neal et al in 1964[12] who suspended a Dacron hub in the abdominal aorta of a pig. The hub was maintained in the center of the lumen by tethering suture lines. Endothelial cells did grow on the hub, but this experiment was criticized because the tethering lines were short and endothelial cells could have crept along them. In an electron microscopic study of rat lungs after intravenous injection of collagen,[13] we found that microscopic thromboemboli of collagen and platelets were promptly covered by thin, flat mononuclear cells that appeared to originate from the blood. However, we could not entirely rule out that some or all of these covering cells derived from the surrounding endothelium. A definitive experiment to prove that blood cells can give rise to endothelium would require the formation of a thrombus on a nonendothelial surface. An experiment that appears to be definitive was published by Feigl et al in 1985:[10] in sheep, a 6-cm segment of the aorta was replaced by a cylinder of impermeable, nonthrombogenic plastic; in the lumen of this tube, a strip of thrombogenic dacron velour was glued transversally so as to form a ring halfway down the tube. This arrangement provided a thrombogenic surface well isolated from contacts with endothelium. The sheep were killed 2 to 84 days later; the velour was found to be covered with a thrombus and eventually with a cellular coat consisting of endothelium over a layer of fibroblasts and myofibroblasts. At that time immunohistochemical markers were not available; however, the authors speculated that stem cells from the blood had colonized the thrombus and provided it with an endothelial cover. Their evidence seems quite conclusive.

Evidence of circulating stem cells became a tangible reality in the 1980s.[14] These cells carry distinctive markers, especially CD34 (which they share with mature endothelial cells) and Flk-1, a receptor for vascular endothelial growth factor.[9] Asahara et al[9] isolated stem cells from human blood by means of magnetic beads coated with anti-CD34 or anti-Flk-1 antibody; when cultured, these cells became spindle-shaped and gave rise to endothelium. Does this mean that stem cells could participate in angiogenesis? To find an answer, Asahara et al[9] labeled the cultured human $CD34^+$ cells with fluorescent acetylated LDL and injected them intravenously into nude mice (to avoid immunological complications) 2 days after the mice had been submitted to unilateral excision of the femoral artery, whereby the operated limb became ischemic. Histology 1 to 4 weeks later showed that many fluorescent cells had become incorporated into vessels, mostly capillaries, in the ischemic limb but not in the normal limb. These fascinating results need to be confirmed, but they certainly open a new window on the cellular mechanisms of angiogenesis.

The origin of fibroblasts from the blood has an equally interesting story, which starts at least as far back as 1902, when Maximow described monocyte-to-fibroblast metaplasia in sterile exudates in rabbits. His conclusions were based on morphology alone and stirred much opposition. Maximow broached the topic again in 1928 by tissue culture, but doubted that "the diehards of orthodox hematology" would accept his new data on monocyte-to-fibroblast metaplasia.[15] In the same years one of the pioneers of tissue culture, Alexis Carrel, reported that he too could culture fibroblasts from blood monocytes, but his methodology is not clear; according to J. K. Moen, also from the Rockefeller Institute, he used explants of chick buffy coat.[16] Moen himself in 1935 prepared cultures of mononuclear cells obtained by irritating the guinea-pig pleura with low-melting point wax, then washing it out 5 to 7 days later. Using Carrel flasks, he followed the growth of individual mononuclear cells, some of which became "typical fibroblast colonies." The illustrations support his contention; however, by today's standards, we cannot exclude that some fibroblasts were present in the starting material.

In the 1930s and 1940s it was the lymphocytes' turn to be considered as precursors of fibroblasts, usually via an indirect route, lymphocyte → monocyte → fibroblast. One opponent of this concept was Peter Medawar.[17] Still, the idea surfaced again in 1959. By that time millipore chambers had come into use; in a careful study, Shelton and Rice[15] collected cells from the normal peritoneum of mice, placed them in diffusion chambers, implanted them in mice or rats, and consistently obtained fibroblasts (as well as collagen, chemically identified). The conclusion:

fibroblasts could derive directly from macrophages and indirectly from lymphocytes via an intermediate large cell that Maximow had called a "polyblast." A flaw in this study is that the authors did not take precautions against the possibility of seeding fibroblasts into the peritoneum when they injected 1 ml of saline intraperitoneally before the collection of cells.

This criticism does not apply to a series of well-controlled studies by Allgöwer and collaborators that began in 1956.[18,19] This group reported that explants of rabbit buffy coat produced collagen *in vitro*. An important refinement was introduced: to exclude the possibility that fibroblasts might be collected inadvertently in the needle used for cardiac puncture, the blood was obtained from cannulated carotid arteries. Of 343 cultures obtained in this fashion, 9% produced collagen. The authors concluded that buffy coat mononuclear cells "are capable of transforming into functional fibrocytes." I can find no fault in this conclusion.

In the meantime Petrakis et al published their experiments on human buffy coats placed in diffusion chambers implanted in human volunteers.[17] After 3 to 4 weeks, typical fibroblasts appeared. In some chambers fat cells developed. It was concluded that mononuclear cells in the blood comprise a "mesenchymal progenitor cell pool." The value of this work is unfortunately diminished, once again, by the lack of controls for accidental sampling of fibroblasts by the intravenous needle, as used by Allgöwer. However, this study included a new twist: a small amount of India ink was added to the chamber content. The ink's carbon particles were present at first in the macrophages and later in the fibroblasts, suggesting that the latter derived from the former.

In 1965, Ross and Lillywhite tested buffy coats once again,[20] comparing samples obtained from guinea pigs by transcutaneous heart puncture to samples obtained from a cannulated carotid artery. Rather than cultures, as used by Allgöwer, they used implanted Millipore chambers. After 28 to 42 days the percentage of chambers containing fibroblasts and collagen was 90.6% for samples obtained by regular transcutaneous cardiac puncture and 6% for samples collected from a cannulated carotid artery.

The authors interpreted these results conservatively. Although fibroblasts (in fact myofibroblasts, according to the description and illustrations) appeared in some chambers of all groups, the authors concluded that their data were, in effect, inconclusive. Interestingly, their percentage of positive cultures from cannulated arteries (6%) was about the same as in the studies of Allgöwer mentioned above (9%). In retrospect, we could argue that both laboratories showed the same thing, namely that fibroblasts can sometimes be obtained from buffy coats; the fact that fibroblasts are not always obtained suggests that the precursor cells are few.

Yet another *in vivo* approach was used by Campbell and Ryan in 1983,[21] who set out to show that peritoneal macrophages could turn into myofibroblasts. They prepared pea-sized blood clots from donor rats and introduced them aseptically into the peritoneal cavity of recipient rats, where most of the implants remained free. After one day the clots were covered with a layer of typical plump "peritoneal macrophages." Then, during the following 2 weeks, the macrophages became flattened and stratified and gave rise to a capsule consisting of typical myofibroblasts. The contractility of this capsule was tested experimentally.[22] The authors concluded that peritoneal macrophages (or a subset of cells mixed with them) can turn into fibroblasts and myofibroblasts. We repeated these experiments in our laboratory and found the results to be very reproducible. Although some contamination with fibroblasts from laparotomy cannot be ruled out, the progressive flattening of plump peritoneal macrophages to thin, elongated myofibroblasts shown by electron microscopy is very convincing.

And finally there came, in 1994, a "molecular" study: Bucala et al[23,24,25] found that blood from humans and mice contains a set of cells ($CD34^+$/$collagen^+$/$vimentin^+$) that migrate out of the blood into areas of wound healing. They gave these cells the unfortunate name of "fibrocytes" (possibly by analogy with monocytes) and concluded that they are an important component of wound healing as a source of cytokines as well as of Type I collagen. Whether these fibroblast-generating stem cells are the same as the above-mentioned endothelium-generating cells is not yet clear; the methods of isolation are not quite the same.

I have dwelt in some detail on these two stories, leading to blood-derived endothelium and blood-derived fibroblasts, because they have a lot in common. Both consist of a crescendo of morphological studies culminating in a solution, followed by a definitive, admittedly exciting molecular study; neither of the molecular papers mentions a single one of the references quoted herein. Even in the computer era, molecular biologists rediscover whole new worlds that had already been carefully explored by morphologists.

To sum up, it is no longer a theory but a fact that blood contains cells capable of producing both endothelium and fibroblasts. Indeed, it is already appropriate to ask the next question: how important are these blood-derived cells? For the macrocirculation, blood-derived endothelial cells could be important for covering a platelet thrombus whereby it would be mechanically stabilized and prevented from growing any more. For the microcirculation, which consists mainly of endothelial cells, a role for blood-derived endothelium is less obvious. The same can be said for blood-derived fibroblasts: in a large vessel they could help stabilize a thrombus (there are few if any fibroblasts in the normal intima), whereas in wound healing, which is played out at the level of the microcirculation, local fibroblasts should be plentiful. Time will tell.

To return to the paper by Thurston et al,[1] a special appeal of their study is the combination of state-of-the-art methods such as lectin-based stains of the endothelium with the whole-tissue approach typical of the 1800s. This three-dimensional method is essential when the object to be studied is a tree-like structure such as the blood vessels. Cohnheim[5] was obliged to study living membranes, such as the mesentery, because thin tissue sections did not exist in his day. This drawback turned out to

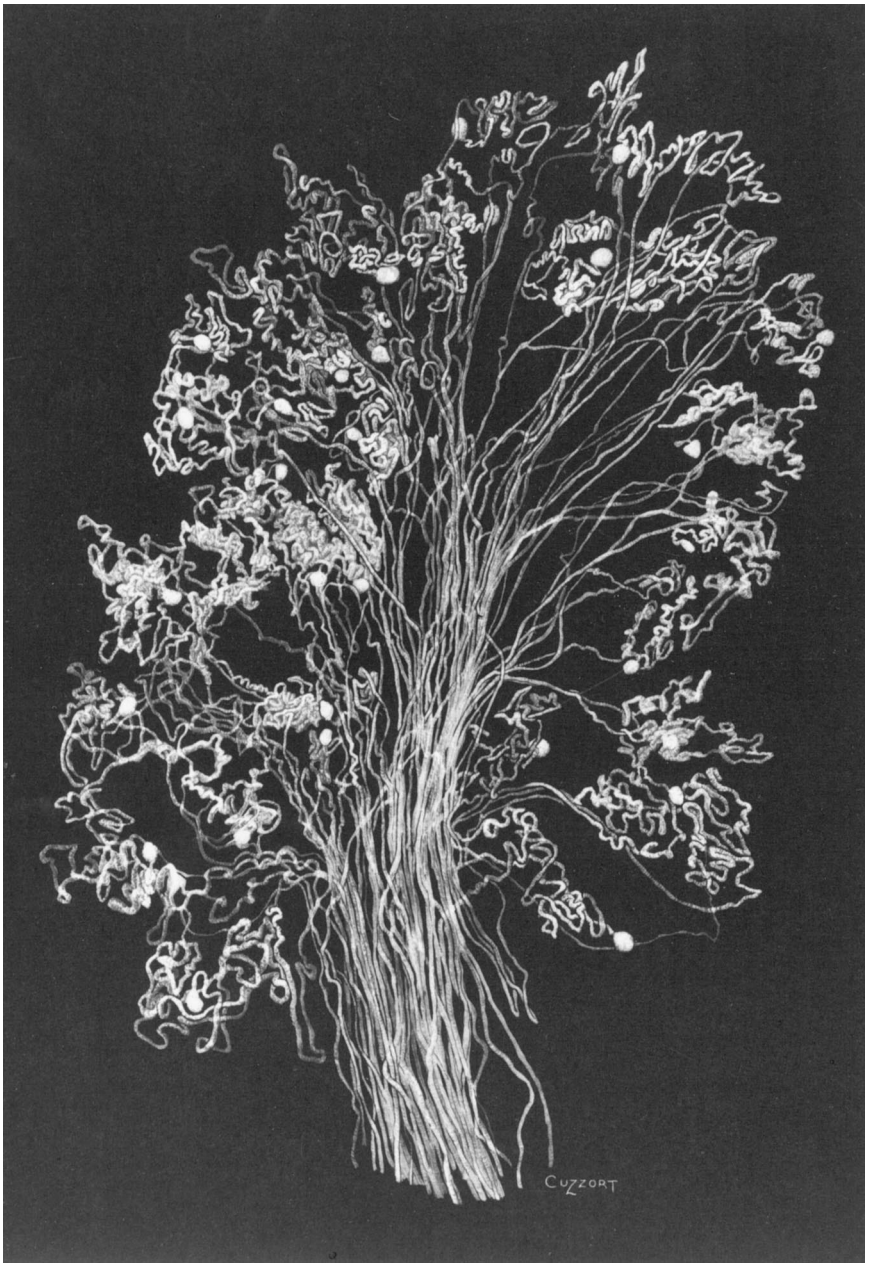

Figure 1. Microdissection of a normal adult kidney, reprinted with permission from Oliver, 1939.[27] Magnification, ×15.

be an advantage because it enabled him to discover the different behavior of arterioles, capillaries, and venules, which in histological sections look very much alike. We have become so accustomed to cross sections that we no longer realize how much we miss in the process; but just imagine the difference between Mona Lisa as Leonardo painted her, and a cross section of his painting—or even of Mona Lisa herself. The loss of information in cross sections is enormous.

Among the examples of the three-dimensional microscopic approach in pathology, the most spectacular is probably the microdissection of single nephrons that allowed Jean Oliver to ask and answer his intriguing question: "When is a kidney not a kidney?"[26] His three-dimensional preparations from end-stage kidneys showed that "no nephron is like its neighbor," a finding as definitive as any DNA gel. I would like to offer Dr. Thurston and his team a bouquet of nephrons (Figure 1)[27] as a special Jean Oliver Award for a careful study that is both informative and beautiful.

References

1. Thurston G, Murphy TJ, Baluk P, Lindsey JR, McDonald DM: Strain-dependent differences in angiogenesis in mice with chronic airway inflammation. Am J Pathol 1998, 153:1099-1112
2. Garcia FU, Wojta J, Broadley KN, Davidson JM, Hoover RL: *Bartonella bacilliformis* stimulates endothelial cells in vitro and is angiogenic in vivo. Am J Pathol 1990, 136:1125–1135
3. Dehio C, Meyer M, Berger J, Schwarz H, Lanz C: Interaction of

Bartonella henselae with endothelial cells results in bacterial aggregation on the cell surface and the subsequent engulfment and internalisation of the bacterial aggregate by a unique structure, the invasome. J Cell Sci 1997, 110:2141–2154
4. Majno G, Joris I: Cells: Tissues and Disease: Principles of General Pathology. Cambridge, MA, Blackwell Science, 1996
5. Cohnheim J: Ueber Entzündung und Eiterung. Virchows Arch (Path Anat) 1867, 40:1–79
6. Boswell CA, Joris I, Majno G: The concept of cellular tone: reflections on the endothelium, fibroblasts, and smooth muscle cells. Perspec Biol Med 1992, 36:79–86
7. Doukas J, Cutler AH, Boswell CA, Joris I, Majno G: Reversible endothelial cell relaxation induced by oxygen and glucose deprivation. Am J Pathol 1994, 145:211–219
8. Joris I, Cuénoud HF, Underwood JM, Majno G: Capillary remodeling in acute inflammation: a form of angiogenesis. FASEB J 1992, 6:A938
9. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T: Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997, 275:964–967
10. Feigl W, Susani M, Ulrich W, Matejka M, Losert U, Sinzinger H: Organisation of experimental thrombosis by blood cells. Virchows Arch (Pathol Anat) 1985, 406:133–148
11. Leu JL, Feigl W, Susani M: Angiogenesis from mononuclear cells in thrombi. Virchows Arch A 1987, 411:5–14
12. O'Neal RM, Jordan GL, Rabin ER, DeBakey MF, Halpert B: Cells grown on isolated intravascular Dacron hub. Exp Mol Pathol 1964, 3:403–412
13. Joris I, Braunstein Jr. PW: Platelets and endothelium: effect of collagen-indudced platelet aggregates on pulmonary vessels. Exp Mol Pathol 1982, 37:393–405
14. Levitt D, Mertelsmann R (eds): Hematopoietic Stem Cells. New York, Marcel Dekker, 1995
15. Shelton E, Rice ME: Growth of normal peritoneal cells in diffusion chambers: a study in cell modulation. Am J Anat 1959, 105:281–341
16. Moen JK: The development of pure cultures of fibroblasts from single mononuclear cells. J Exp Med 1935, 61:247–263
17. Petrakis NL, Davis M, Lucia SP: The in vivo differentiation of human leukocytes into histiocytes, fibroblasts, and fat cells in subcutaneous diffusion chambers. Blood 1961, 17:109–118
18. Allgöwer M, Hulliger L: Origin of fibroblasts from mononuclear blood cells: a study on in vitro formation of the collagen precursor, hydroxyproline, in buffy coat cultures. Surgery 1960, 47:603–610
19. Hulliger L, Allgöwer M: Monocyte participation in connective tissue repair. Experientia 1963, 19:577–580
20. Ross R, Lillywhite JW: The fate of buffy coat cells grown in subcutaneously implanted diffusion chambers. Lab Invest 1965, 14:1568–1585
21. Campbell GR, Ryan GB: Origin of myofibroblasts in the vascular capsule around free-floating intraperitoneal blood clots. Pathol 1983, 15:253–264
22. Ryan GB, Cliff WJ, Gabbiani G, Irlé C, Statkov PR, Majno G: Myofibroblasts in an avascular fibrous tissue. Lab Invest 1973, 29:197–206
23. Bucala R, Spiegel LA, Chesney J, Hogan M, Cerami A: Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. Mol Med 1994, 71–81
24. Chesney J, Bacher M, Bender A, Bucala R: The peripheral blood fibrocyte is a potent antigen-presenting cell capable of priming naive T cells in situ. Proc Natl Acad Sci USA 1997, 94:6307–6312
25. Chesney J, Metz C, Stavitsky AB, Bacher M, Bucala R: Regulated production of type I collagen and inflammatory cytokines by peripheral blood fibrocytes. J Immunol 1998, 160:419–425
26. Oliver J: When is the kidney not a kidney? J Urol 1950, 63:373–402
27. Oliver J: Architecture of the Kidney in Chronic Bright's Disease. New York, Paul B. Hoeber, Inc, 1939